

Genetic Loci of Major Antigenic Protein Genes of *Edwardsiella tarda*

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Seven antigenic proteins of *Edwardsiella tarda* were identified by using a rabbit polyclonal antiserum. Four of these proteins also reacted with a Japanese flounder antiserum. The amino acid sequences had identity to lipoproteins, periplasmic proteins, and exported and secreted proteins with roles in transport of metabolites across the cell membrane, stress response, and motility. These genes and their products are useful for developing DNA or recombinant subunit vaccines to control edwardsiellosis.

Edwardsiella tarda is a gram-negative bacterium that induces disease mostly in fish (33) but also in humans (18). The disease in fish, termed edwardsiellosis, is characterized by skin lesions that progress into suppurative abscesses, hyperemia, hemorrhages, swelling, and necrosis, all of which are disseminated systemically (29, 33). Among a number of factors related to *E. tarda* virulence (5, 14, 26, 34), only hemolysin has been associated with invasion and cytotoxicity (17, 38). At present, the pathogenesis of the disease, as well as the antigenic proteins that could induce protective immunity, is unknown.

In this study, we constructed a cosmid DNA library of *E. tarda* 54, a virulent strain isolated from an outbreak of disease in Japanese flounder (*Paralichthys olivaceus*). The library was screened for clones expressing antigenic proteins using a rabbit anti-*E. tarda* (NG8104) polyclonal antiserum as the first antibody and a goat anti-rabbit immunoglobulin G (IgG) bound to ^{125}I as the second antibody. Six different cosmid clones were found and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses (24). The proteins were transferred to polyvinylidifluoride membranes and then incubated with the same first antibody. A goat anti-rabbit IgG conjugated with alkaline phosphatase was used as the second antibody (Fig. 1). The immune reaction was revealed with the 5-bromo-4-chloro-3-indolyl phosphate-Nitro Blue Tetrazolium substrate.

Cosmid DNA from each clone was digested with several restriction enzymes, and the DNA fragments were cloned in plasmid vectors for immunoscreening and sequencing. IPTG (isopropylthio- β -D-galactoside) at 1 mM was added to induce expression of the protein. Nucleotide sequences were determined by the cycle sequencing method using Thermo Sequenase. Then, specific primers were designed to amplify the putative open reading frames (ORFs) and each gene was cloned into pBluescript SK(+) vector. Recombinant proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with the same antibodies (Fig. 2). The proteins were also analyzed by using a Japanese

flounder serum raised against the bacteria (first antibody), a rabbit polyclonal antiserum against Japanese flounder IgM as the second antibody, and the goat anti-rabbit IgG conjugated with alkaline phosphatase as the third antibody (Fig. 3). All sera used in this study were previously incubated with the *Escherichia coli* strains carrying empty vectors to remove unspecific antibodies. DNA preparations were carried out by using standard procedures (37). Sequence data were compared with those in GenBank by using the BLAST program. Putative signal peptides were determined by using the Signal P program (4). The proteins were named Et 18 through Et 76 based on their molecular masses (Table 1).

Et 18 and Et 28 (putative lipoproteins), together with Et 76, were expressed in clones 54-3 and 54-4 (Fig. 1 and 4). Their amino acid sequence identities are compared in Table 2. Et 18 had identity to *Salmonella enterica* periplasmic lipoprotein (YraP) (31), whereas Et 28 had identity to *Shigella flexneri* and *E. coli* putative lipoprotein (YaeC) (3, 20). Both proteins possess signal peptides at the N terminus and the consensus cleavage site {LA(G,A) ↓ C} of bacterial lipoproteins for peptidase II, where the arrow indicates the position of cleavage between

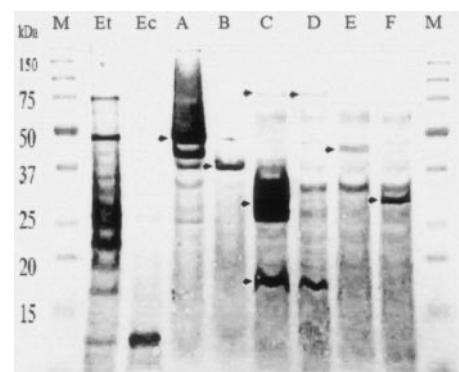


FIG. 1. Western blot analysis of total cell proteins of six *Escherichia coli* cosmid clones expressing *Edwardsiella tarda* antigenic proteins. Lane M, marker; lane Et, *E. tarda* strain 54; lane Ec, *E. coli* XL1-Blue MR carrying SuperCos I vector; lane A, cosmid 54-1 carrying the Et 49 gene; lane B, cosmid 54-2 carrying the Et 38 gene; lane C, cosmid 54-3 carrying the Et 18, Et 28, and Et 76 genes; lane D, cosmid 54-4 carrying the Et 18 and Et 76 genes; lane E, cosmid 54-5 carrying the Et 46 gene; lane F, cosmid 54-6 carrying the Et 32 gene. Arrows indicate the antigenic protein band.

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TABLE 1. General features of the *Edwardsiella tarda* antigenic proteins and oligonucleotide primers used for the amplification of each ORF

ORF (bp)	Protein name	Immature protein size (kDa)	No. of amino acids	Mature protein size (kDa)	No. of amino acids	Homologous protein	Primer sequences ^a
591	Et18	2047	196	17.8	172	Putative periplasmic lipoprotein (YraP)	(F) 5'-ATACCCCTGTTCTTCACCAG3' (R) 5'TTTGGCTATTGCGGTGGG3'
816	Et28	29.38	271	27.24	249	D-Methionine binding lipoprotein (YaeC)	(F) 5'AGCGTGAGACACAACAT3' (R) 5'GCAATGACAAGATGGCGA3'
951	Et32	33.86	316	31.63	294	Putative exported protein (YPO2305)	(F) 5'CTTCTGCGCTAACCAAAC3' (R) 5'GGGGCTTATTAAACAG3'
1194	Et38	43.49	397	40.98	371	Periplasmic maltose binding protein (MalE)	(F) 5'TAAACTCCCCGACAGGACTAC3' (R) 5'TTAATACGGTGTAGAGCCCG3'
1251	Et46	43.8	416	43.8	416	Flagellin (FlC)	(F) 5'TAGAAGCAGGAAAATGGGG3' (R) 5'ACGCCGTTAATGCCGATTAAACG3'
1416	Et49	48.8	471	46.2	445	Global stress requirement protein (GsrA)	(F) 5'GGGATATATCGTGTACGGGTATTG3' (R) 5'AATACGTCACGGGGTATTG3'
2154	Et76	76.76	717	76.76	717	Glycosylase (Yram), putative exported protein	(F) 5'CAGAAATGGCAGCATGATCG3' (R) 5'AATGCATGGCAGCCATTG3'

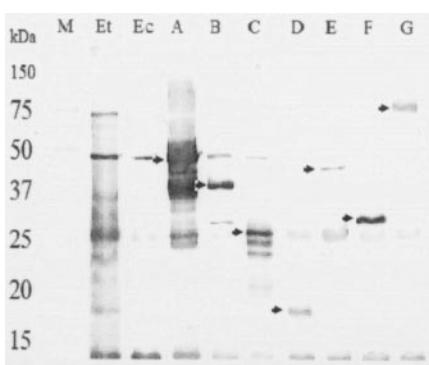
^a F, forward; R, reverse.

FIG. 2. Western blot analysis of *Edwardsiella tarda* recombinant antigenic proteins reacting with a rabbit serum anti-*E. tarda*. Lane M, marker; lane Et, *E. tarda* strain 54; lane Ec, *Escherichia coli* JM109 carrying pBluescript vector; lane A, *E. coli* carrying the Et 49 gene; lane B, *E. coli* carrying the Et 38 gene; lane C, *E. coli* carrying the Et 28 gene; lane D, *E. coli* carrying the Et 18 gene; lane E, *E. coli* carrying the Et 46 gene; lane F, *E. coli* carrying the Et 32 gene; and lane G, *E. coli* carrying the Et 76 gene. Arrows indicate the antigenic protein band.

the signal peptide and the mature protein, the amino acid cysteine is absolutely required in position +1, alanine, glycine, or serine is acceptable in position -1, and alanine and leucine are preferred in positions -2 and -3, respectively (12). Bacterial lipoproteins are one of the components of pathogen-associated molecular patterns produced by microorganisms (19). They possess immunostimulatory activities, i.e., they trigger the innate immune response via Toll-like receptors 2 and 6 (TLR2 and TLR6) in mammals (1, 15, 39). In all cases, a tripalmitoyl-S-glyceryl-modified cysteine (Pam_3Cys) is required for these stimulatory properties (8, 44). Japanese flounder also possess a functional homolog of the human TLR2 (13). Therefore, it is reasonable to assume that the immunostimulatory properties of these proteins in Japanese flounder and human are similar. The genetic location of the Et 28 gene in the chromosome (Fig. 4) was the same as that reported for

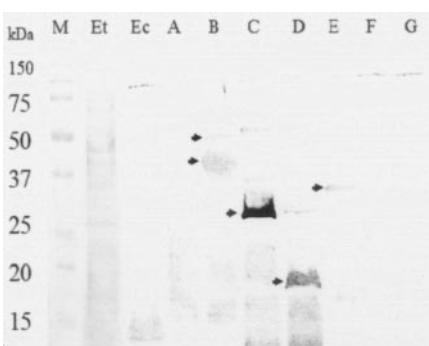
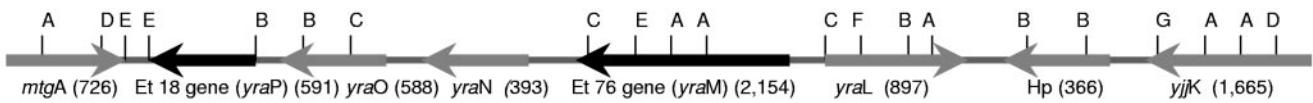
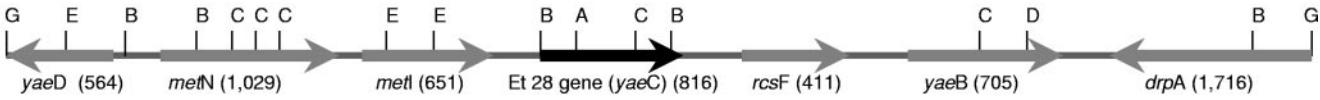


FIG. 3. Western blot analysis of *Edwardsiella tarda* recombinant antigenic proteins reacting with a Japanese flounder anti-*E. tarda* serum. Lane M, marker; lane Et, *E. tarda* strain 54; lane Ec, *Escherichia coli* BL21 codon plus carrying pET32 expression vector; lane A, *E. coli* carrying the Et 49 gene; lane B, *E. coli* carrying the Et 38 gene; lane C, *E. coli* carrying the Et 28 gene; lane D, *E. coli* carrying the Et 18 gene; lane E, *E. coli* carrying the Et 32 gene; lane F, *E. coli* carrying the Et 46 gene; lane G, *E. coli* carrying the Et 76 gene. Arrows indicate the antigenic protein band.

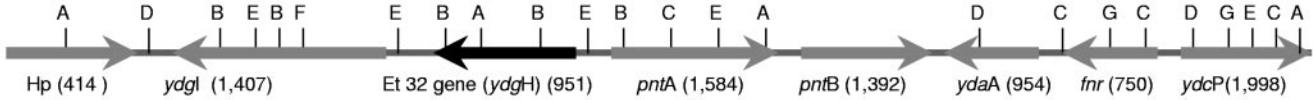
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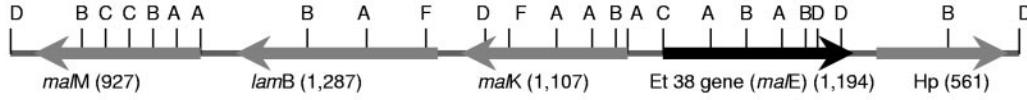
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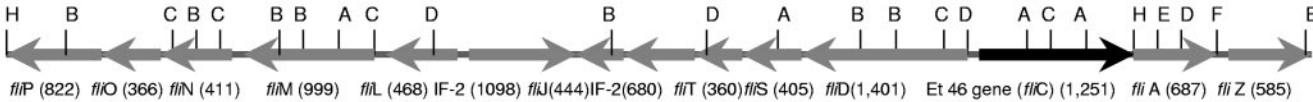
Clone 54-6



Clone 54-2



Clone 54-5



Clone 54-1

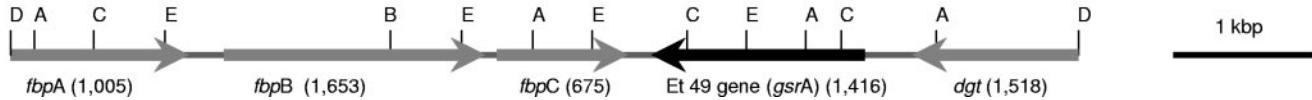


FIG. 4. Schematic representation of the genetic loci of major antigenic proteins of *Edwardsiella tarda*. Open reading frames (ORFs) and the transcription directions are indicated by arrows. Sizes of ORFs in base pairs are in parentheses. Black arrows indicate antigenic protein genes. Restriction sites are as follows: A, PstI; B, HincII; C, EcoRV; D, BamHI; E, SacII; F, SacI; G, EcoRI; and H, HindIII. Gene names and product are as follows: *dgt*, deoxyguanosine triphosphate triphosphohydrolase; *drpA*, prolyl-tRNA synthetase; *fbpA*, ferric binding protein A; *fbpB*, transmembrane permease (iron III); *fbpC*, ABC transporter, ATP binding protein; *fli4*, RNA polymerase, regulator of flagellar operon, sigma factor 28; Et 46 gene (*fliC*), flagellin FliC; *fliD*, flagellar capping protein, hook-associated protein 2; *fliJ*, flagellar protein FliJ; *fliL*, flagellar biosynthesis protein FliL; *fliM*, flagellar motor switch protein FliM; *fliN*, flagellar motor switch protein FliN; *fliO*, flagellar protein fliO; *fliP*, flagellar biosynthesis protein FliP; *fliS*, flagellin-specific chaperone FliS; *fliT*, repressor of class 3a and 3b operon FliT; *fliZ*, regulator of FliA; *fnr*, fumarate and nitrate reduction regulatory protein; Et 49 gene (*gsrA*), global stress requirement protein A; *lamB*, maltoporin LamB; Et 38 gene (*malE*), maltose binding periplasmic protein MalE; *maK*, maltose ATP binding protein MaK; *maM*, maltose binding protein MaM; *metN*, D-methionine transport, ATP binding protein MetN; *mtgA*, monofunctional biosynthetic peptidoglycan transglycosylase; *pntA*, pyridine nucleotide transhydrogenase, alpha subunit; *pntB*, pyridine nucleotide transhydrogenase, beta subunit; *rcsF*, regulator of colanic acid synthesis RcsF; *yaeB*, hypothetical protein YaeB; Et 28 gene (*yaeC*), putative lipoprotein YaeC; *yaeD*, putative phosphatase YaeD; *ydaA*, universal stress protein YdaA; *ydcP*, putative protease, collagenase YdcP; Et 32 gene (*ydgH*), putative exported protein; *yjjK*, ATPase component of ABC transporter YjjK; *yraL*, putative tetrapyrrole methylase YraL; Et 76 gene (*yraM*), putative glycosylase YraM; *yraN*, hypothetical protein, endonuclease YraN; *yraO*, probable phosphosugar isomerase YraO; Et 18 gene (*yraP*), possible lipoprotein YraP. Hp, hypothetical protein; IF-2, translation initiation factor 2.

S. flexneri and was designated an O-island (O-island no. 4) in *E. coli* O157 (32). These antigenic protein genes and others genes at the same locus, for instance, the arylsulfate sulfotransferase gene, should be overexpressed during the infection process in fish, just as some *Vibrio vulnificus* genes (*yaeC*) are overexpressed during the infection process in humans (22). The arylsulfate sulfotransferase gene has been described as a potential *E. tarda* virulence gene (26). Thus, this locus could represent a pathogenic island in *E. tarda*.

Et 32 and Et 76 (putative exported proteins) amino acid sequences had identities to *Yersinia pestis* putative exported protein YPO2305 and the glycosylase YraM (Table 2) (6, 30).

The genetic location of the Et 32 gene suggests that it is a component of an operon involved in arginine or pyrimidine nucleotide uptake (Fig. 4). This observation agrees with the expression of a gene (*pyrH*) involved in the synthesis of pyrimidine nucleotides during infections by *V. vulnificus* in humans (22). This pathway is linked to the synthesis of arginine (21).

As mentioned above, Et 76, Et 18, and/or Et 28 was expressed by two clones carrying overlapping DNA fragments. The genetic location (Fig. 4) is the same as that reported in *Y. pestis* (30). Although their functions are unknown, bacterial cell envelope-associated and secreted proteins are immunogenic, with roles in pathogenesis. These proteins comprise 6 to

TABLE 2. Comparison of predicted amino acid sequence identities of *Edwardsiella tarda* antigenic proteins and their homologues in other bacteria

Closely related species	Amino acid sequence identity (%) with:							Reference
	Et 18 (YraP)	Et 28 (YaeC)	Et 32 (YdgH)	Et 38 (MalE)	Et 46 (FliC)	Et 49 (GsrA)	Et 76 (YraM)	
<i>Edwardsiella tarda</i>				95				40
<i>Yersinia enterocolitica</i>					76			45
<i>Yersinia pestis</i>	72	80	62	82	75	65	65	30
	66					65		6
<i>Escherichia coli</i>	71			84		74		32
		81			56		56	3
			55					41
<i>Shigella flexneri</i>	70	81	55	83		74	56	43
<i>Salmonella enterica</i> serovar Typhi CT18	74	80	55	83		73	58	20
<i>Photorhabdus luminescens</i>	63	76	56	75	58		53	31
<i>Serratia marscencens</i>					64			27
								7
								10

37% of the gram-positive proteomes and 12 to 36% of the gram-negative proteomes (36). Secreted and exported proteins are considered important virulence factors and vaccine candidates. Therefore, we believe these genes and proteins may be useful to vaccine development.

Et 38 and Et 49 (putative periplasmic proteins). The Et 38 amino acid sequence had 84% identity to that of the periplasmic maltose binding protein (MalE) of *E. coli* (32), which is involved in energy-dependent translocation of maltose through the cytoplasmic membrane (16). The Signal P program predicted a 26-residue signal peptide, which would produce a mature protein of 40.9 kDa. This size did not correlate well with the antigenic band detected by Western blotting (Fig. 1 and 2). The fish serum reacted with two antigenic bands that might correspond to the immature and mature proteins (Fig. 3). The differences in protein size may be due to an inaccurate prediction of the signal peptide cleavage site.

The Et 49 gene nucleotide sequence had 76% identity to that of the gene for the global stress requirement protein (GsrA) of *Yersinia enterocolitica* (45). The protein conserved the major features of heat shock proteins (HSPs), such as a putative signal peptide, a Ser/His/Asp active site, and a sequence similar to the σ^E type heat shock promoter consensus (35). HSPs preserve cellular functions and are necessary for growth and survival during exposure to environmental stress, including the intramacrophagic state of pathogens such as *Y. enterocolitica* (45) and *Brucella abortus* (2, 23). HSPs are immunogens that induce both cellular and humoral immunity (2). *E. tarda* is considered an intracellular pathogen. It survives and replicates in phagocytes (34). It also grows on media with 3% NaCl and at temperatures as high as 40°C (33). Thus, it was not unexpected that the *E. tarda* heat shock proteins have immunostimulatory activity in a mammalian host. On the other hand, because the flounder serum did not react with this protein, it may not have the same role in fish.

Et 46 (flagellin, a secreted protein). Flagellins (H antigens) belong to the pathogen-associated molecular pattern that, in mammals, activates the innate immune system via TLR5 (11, 25). Bacterial adhesion to and invasion of animals' cells are necessary for virulence and depend on the presence of flagel-

lum in *Aeromonas hydrophila* (28) and *S. enterica* (42). *E. tarda* flagellin was described as an extracellular virulence-associated protein (40). As expected, the *E. tarda* flagellin of this study had 95% amino acid identity with the *E. tarda* flagellin reported by Tan et al. (40). On the other hand, it had moderately low identity (64%) to other bacterial flagellins, particularly in the antigenic domain (Table 2). This domain is a nonconserved region and is very different in bacteria belonging to different species (9). The protein probably does not have strong immunostimulatory functions in fish, since it was recognized by only the rabbit antiserum (Fig. 1 and 2).

Finally, based on the sequence identities and other bioinformatics tools, the *E. tarda* major antigenic proteins are normal components of the bacterial cell wall and are involved in aromatic amino acid, sugar, and probably nucleotide uptake, stress response, and motility. Some *E. tarda* antigenic proteins in the mammal immune system may not be in the fish counterpart. These genes and their products are useful for developing DNA or recombinant subunit vaccines to control edwardsiellosis.

Nucleotide sequence accession numbers. The sequences for the genes encoding Et 18, Et 28, Et 32, Et 38, Et 46, Et 49, and Et 76 were deposited in GenBank under the accession numbers AB195503, AB195504, AB195505, AB195506, AB195507, AB195508, and AB195509, respectively.

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